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Effect of Laser Welding With Human Serum Albumin on the Expression of P-Selectin on Platelets

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Background and Objective: **Artery repair by means of laser energy induces activation of platelets with a risk of thrombosis and local inflammatory reactions. The aim of this study was to investigate the effect of human serum albumin, the most common solder in laser surgery, on platelet activation.**
Study Design/Materials and Methods: **Platelet activation was evaluated in canine blood by using two-color flow cytometry with a phycoerythrin-labeled antibody to a common platelet marker, glycoprotein IIb/IIIa and fluorescein isothiocyanate–labeled antibody to a platelet activation molecule, P-selectin. Human serum albumin was applied in vitro and in vivo, as a solder during laser reconstruction of canine arteries.**
Results: **In vitro, albumin significantly ($P < 0.01$) reduces the expression of P-selectin on platelets. This is most likely related to the blockage of P-selectin by albumin, which binds to the platelet surface, as confirmed by flow cytometry with fluorescein isothiocyanate–labeled albumin. In vivo, application of albumin solder tended to result in a lower percentage of P-selectin–expressing platelets in laser-repaired arteries compared to suture-repaired arteries.**
Conclusion: **Albumin decreases the percentage of P-selectin–expressing platelets in vitro. Further research may allow the platelet activation inhibiting properties of albumin to be further optimized in vivo. Lasers Surg. Med. 25:438–444, 1999.**

Key words: albumin; flow cytometry; laser surgery; platelet activation; P-selectin

INTRODUCTION

Human albumin is known to prevent adhesion of platelets to its coated surface [1,2]. It also inhibits formation of platelet-platelet homoaggregates [3]. Therefore, albumin becomes a promising biomaterial for the treatment of blood vessels during their surgical reconstruction. Thus, albumin used as a solder to strengthen bonds in laser tissue welding [4–6] may also decrease platelet associated complications in laser reconstruction of blood vessels.

Application of laser energy for end-to-end anastomosis of arteries can be accompanied by a severe inflammatory reaction [7]. Recruitment of inflammatory cells to the site of vascular injury is

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known to be mediated by platelets [8–10]. Specifically, certain surface molecules regulate the interaction between platelets and inflammatory cells, granulocytes, and monocytes. These molecules become expressed on the surface of platelets after their activation [11].

A well-studied platelet activation antigen, P-selectin, is a platelet granule protein which upon activation undergoes surface translocation [12]. P-Selectin is a molecule that mediates the interaction between platelets and granulocytes or monocytes followed by the formation of heteroaggregate complexes [12]. These complexes may not only induce local inflammatory reactions but also exacerbate thrombotic events [13].

Albumin has been shown to prevent platelet-platelet interaction and adhesion [2,3]. However, its effect on activation molecules mediating platelet-leukocyte interactions is not clear. In this study, we report the effect of albumin on platelet surface P-selectin expression.

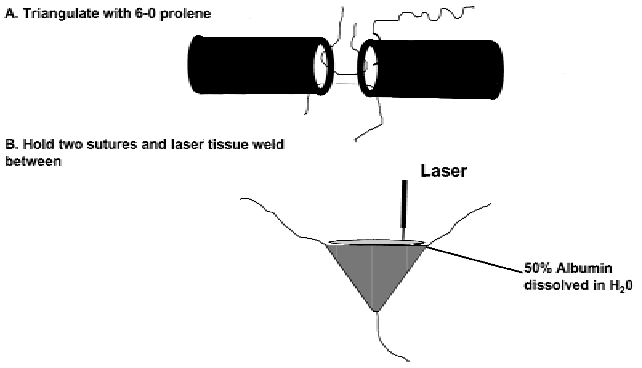

Fig. 1. Preparation of artery for anastomosis by using laser and albumin. **A:** The vessel is triangulated with 6-0 Prolene. **B:** Two of the sutures are held, albumin is applied at the surface, and the vessel is welded.

MATERIALS AND METHODS

Animals

Six mongrel female dogs, each weighing 15–20 kg, were purchased from Taconic (Germantown, NY) and maintained at the Cornell University Medical College Animal Resource Facilities. All animal experiments were carried out according to the protocols approved by the Animal care Committee. The dogs were fasted the night before surgery.

In Vitro Assay

Samples of canine venous blood (1 drop) were collected into tubes containing 3.8% sodium citrate solution in Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, MO) by using a 20-gauge needle with or without a syringe. Fifty-microliter blood aliquots were immediately transferred into tubes containing either 6% fatty acid free human serum albumin (Vitex, New York, NY) in HBSS or HBSS alone and incubated for 60 minutes. After incubation, blood cells were fixed in 1% paraformaldehyde, washed, stained with antibodies, and analyzed by using flow cytometry.

In Vivo Study

The dogs were sedated, anesthetized with telazol (10 mg/kg i.v., Fort Dodge Laboratories, Inc., Fort Dodge, IA) and maintained on isoflurane anesthesia (0.5–2.0 vol % in oxygen). The anterior neck of the animal was shaved from chin to sternum, prepared with providone-iodine (Purdue Frederick Company, Norwalk, CT) and 70% isopropyl alcohol (Clark Surgical Corporation) and then draped in a sterile manner. A 6-cm-long incision was made on the right, 2 cm parallel to the trachea. The right carotid artery was exposed and dissected free from the periadventitial tissue. Subsequently, by using a 20-gauge needle, blood samples were collected into tubes containing 3.8% sodium citrate/1% paraformaldehyde solution. Vascular clamps were applied both proximally and distally to the puncture site and the artery was transected. The artery was irrigated with heparinized saline (Elkins-Sinn, Inc., Cherry Hill, NJ) and reconstructed with either a microsuture technique by using 6-0 polypropylene or with the 1.32 Nd:YAG laser and 50% albumin solder (Vitex, New York, NY). A flash lamp-pumped Nd:YAG laser (Premier Lasers Systems, Inc., Irvine, CA) was used to perform an end-to-end arterial anastomosis. The arterial edges were approximated end to end, without eversion before laser welding, as shown in Figure 1. The laser was used in conjunction with a protein solder (50% human albumin, 50% distilled water by weight). The solder (0.4 ± 0.2 ml) was applied onto the edges of the triangulated arterial walls (Fig. 1). The laser operated in a continuous mode at a wavelength of 1.32 μm and power of 2 ± 0.2 W. The beam was delivered by a silica fiber with a core diameter of 400 μm.

The clamps were removed, and the neck wound was closed in layers. The same procedure was repeated on the left side. After 2 hours, the wounds were reopened and carotid arteries were reexposed. Fifty-microliter samples of blood were

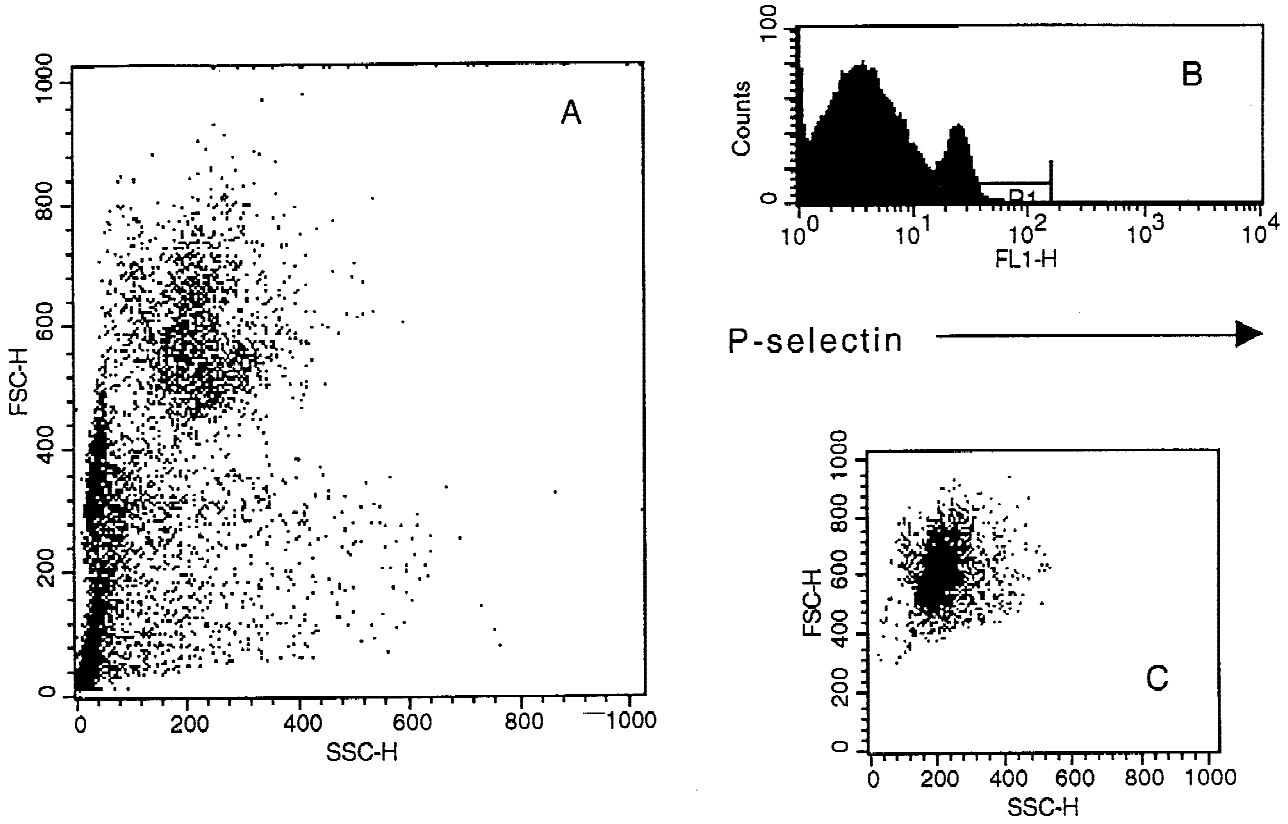

Fig. 2. P-Selectin positive cells appear on SS/FS in a site typical for granulocytes and monocytes. **A:** Total blood cells were stained with fluorescein isothiocyanate–labeled antibody to P-selectin. **B:** P-selectin–positive cells were gated. **C:** The location of P-selectin–positive cells on FS/SS is shown. SS, side scatter pattern; FS, forward scatter pattern; FSC, forward light scattering direction; SSC, side light scattering direction.

drawn from sites that were 1 cm proximal and 1 cm distal to the site of anastomosis. The distal site sample was collected initially in all samples.

Flow Cytometry

Washed blood cells were adjusted to a 100-μl volume in HBSS containing 5% fetal calf serum (Sigma) and the following antibodies were applied: fluorescein isothiocyanate (FITC) –labeled anti-human CD62P (P-selectin) (Pharmingen, San Diego, CA) and phycoerythrin-labeled anti-human CD41 (glycoprotein [GP] IIb/IIIa) (DAKO, Carpinteria, CA). These antibodies cross-reacted with canine antigens, P-selectin, and GP IIb/IIIa, respectively [14,15]. Binding of albumin to the platelets has been verified by the addition of FITC-labeled albumin (Sigma) in the final concentration of 12.5 mg/ml to total blood cells. As a control, streptavidin-FITC (Sigma) has been used. After incubation, cells were washed three times in HBSS with 5% fetal calf serum and analyzed by flow cytometry by using FACScan (Becton Dickinson, Franklin Lakes, NJ) and CellQuest software. Ten thousand electronically gated cells, based on the forward-scatter (FS) and side-scatter (SS) pattern, were analyzed per sample.

Statistical Analysis

The results are expressed as mean ±SD. The data have been analyzed by using Student's t-test, and results were considered significant at $P < 0.05$.

RESULTS

P-Selectin Expression

Total blood cells were used in flow cytometry analysis (Fig. 2A). When P-selectin–positive cells were electronically gated (Fig. 2B) and analyzed for light scattering in forward (FSC) and side (SSC) direction, they appeared in a region typical for granular cells and monocytes (Fig. 2C). This region may also contain platelets in complexes with granulocytes and monocytes. Therefore, we

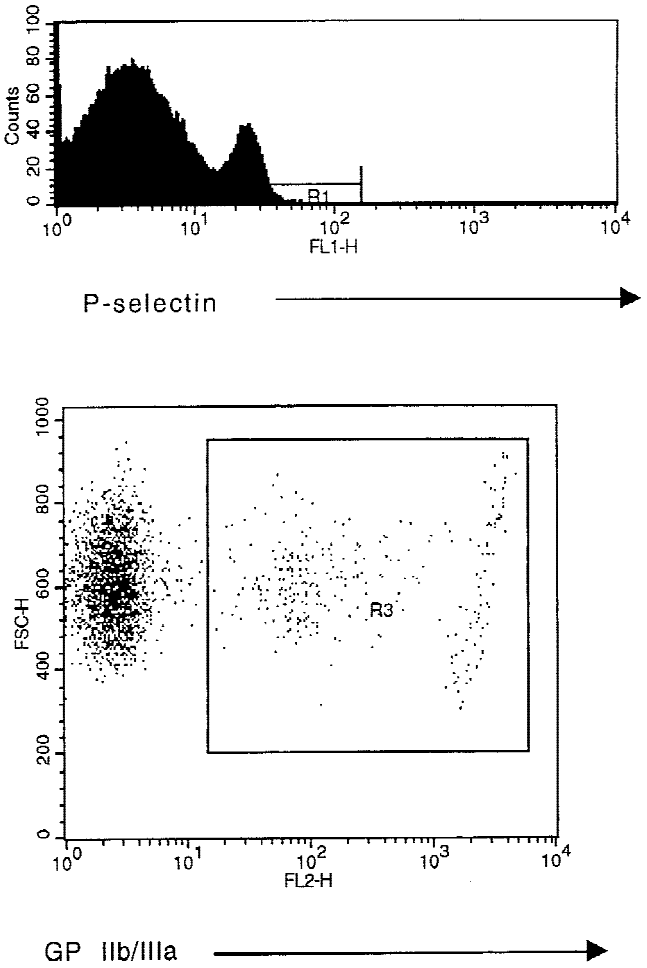

Fig. 3. P-Selectin–positive platelets were distinguished from P-selectin–positive leukocytes by their surface expression of glycoprotein (GP) IIb/IIIa. Total blood cells were stained with the fluorescein isothiocyanate–labeled antibody to P-selectin (Fl1) and phycoerythrin-labeled antibody to GP IIb/IIIa (Fl2). P-Selectin–positive cells were gated, R1 (top) and analyzed for GP IIb/IIIa expression (bottom). GP IIb/IIIa-positive cells (R3) are platelets in complexes with large cells, granulocytes and monocytes, according to their location on light scattering in forward. FSC, forward light scattering direction.

examined this population for the expression of GPIIb/IIIa, the platelet marker. Region R3 of Figure 3 represents all GPIIb/IIIa-positive cells, i.e., platelets. However, the high position of region R3 on FSC suggests that these P-selectin–positive platelets are in complexes with monocytes and granulocytes.

Effect of the Blood Drawing Procedure

We further studied whether a blood-collection procedure has any effect on platelet P-selectin expression. We found that platelets obtained by needle puncture showed a detectable

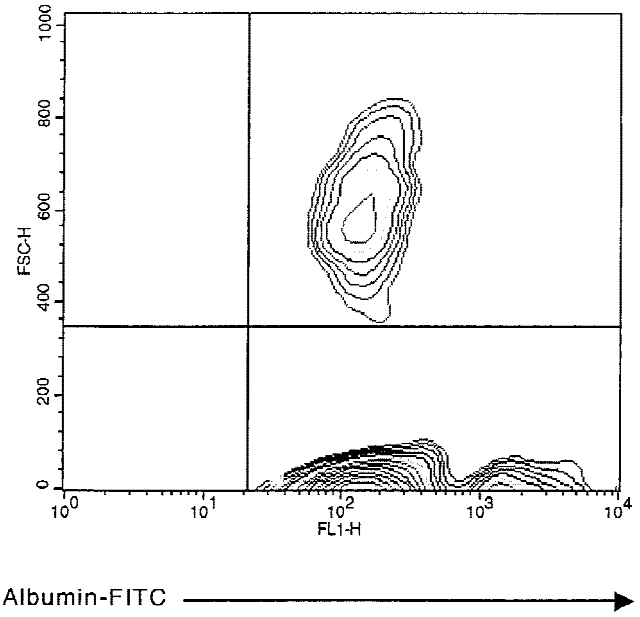

Fig. 4. All platelets bind albumin. Total blood cells were stained with fluorescein isothiocyanate-labeled albumin and phycoerythrin-labeled antibody to glycoprotein (GP) IIb/IIIa. GP IIb/IIIa-positive cells were analyzed for albumin binding and size. Free platelets appear low on forward-scatter pattern, whereas leukocyte-associated platelets show high forward light scattering. Quadrant settings are adjusted according to the negative controls.

expression of P-selectin on their surface with an average of 5.4% of platelets being P-selectin positive. The percentage of P-selectin–positive platelets was slightly higher in blood samples drawn with a syringe compared with the samples obtained by needle puncture alone (Table 1), but this percentage was not statistically significant.

In Vitro Effect of Albumin

The expression of platelet surface molecules might be affected by albumin if it binds to the platelet surface. To verify this, we have used FITC-labeled albumin. Flow cytometry analysis of cells incubated with albumin-FITC demonstrated its binding to virtually all platelets, both free and in complexes with leukocytes according to their FS profile (Fig. 4). Incubation of platelets with fatty acid free human serum albumin resulted in a significant decrease in the percentage of P-selectin–positive platelets (Table 1; Fig. 5C).

In Vivo Effect of Albumin

The expression of P-selectin on platelets was comparable in all animals before arterial repair (Table 2). It significantly increased after the re-

TABLE 1. The Effect of Blood Drawing Procedure on Platelet P-Selectin Expression

Blood sampling handling	P-Selectin positive platelets, %
Aspiration with a syringe	8.0 ± 2.2
Needle puncture	5.4 ± 1.8
Needle puncture followed by incubation with albumin	0.6 ± 0.5*

*P < 0.01 compared to simple needle puncture.

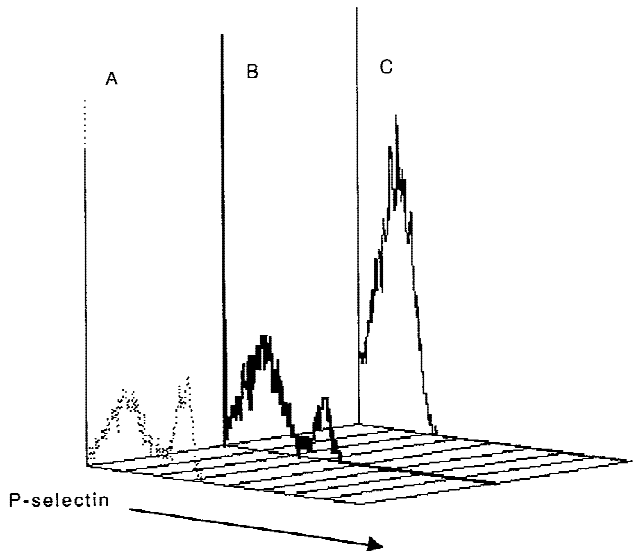

Fig. 5. The percentage of P-selectin–expressing platelets decreases after exposure to albumin. Platelets from control suture-repaired arteries, distal end (**A**), albumin-laser–repaired arteries, distal end (**B**) and samples treated in vitro with albumin (**C**) were compared for P-selectin expression by using the antibodies (anti–P-selectin fluorescein isothiocyanate and anti-glycoprotein [GP] IIb/IIIa) and flow cytometry. Platelets gated by GP IIb/IIIa expression show a P-selectin–positive subset in control (A) and a smaller subset, i.e., in in vivo treated samples (B) but not in in vitro treated samples (C).

pair. The percentage of activated platelets reached peak numbers at the distal end of the anastomosis, where it tended to be higher in suture ($P < 0.001$ compared with initial data) than albumin-laser ($P < 0.01$ compared with initial data) group without reaching statistical significance (Table 2; Fig. 5A,B). The percentage of P-selectin–expressing platelets at the distal end of the anastomosis was significantly higher than at the proximal end in suture, but not in albumin-laser group ($P < 0.05$). In both groups, the percentage of P-selectin–positive platelets was significantly higher after than before reconstruction ($P < 0.05$) (Table 2).

To assess whether albumin would be in contact with the luminal surface of the artery, we prepared histologic specimens of the wound site. Analysis of these sections revealed that albumin is found both at the adventitial and luminal surfaces of the artery (Fig. 6).

TABLE 2. A Comparison of P-Selectin Expression on Platelets After Arterial Repair Using Albumin-Laser or Suture Reconstruction

	P-Selectin expressing platelets	
Blood sampling	Suture	Albumin-laser
Before reconstruction	6.3 ± 3.0	6.4 ± 3.6
After reconstruction at proximal end	10.3 ± 2.5*	14.6 ± 4.8*
After reconstruction at distal end	21.5 ± 6.1***†	16.8 ± 3.4**

*P < 0.05 compared to the initial data
**P < 0.01 compared to the initial data
***P < 0.001 compared to the initial data
†P < 0.05 compared to the proximal end

DISCUSSION

In blood vessel reconstruction, platelet activation can lead to thrombogenesis and local inflammatory reactions that significantly compromise the repair [16]. This can be prevented by monoclonal antibodies to P-selectin [17,18]. In laser surgery of blood vessels, which is known to induce platelet activation and aggregation [19,20], platelet activation inhibiting agents become extremely important. Interestingly, human serum albumin used as a solder in laser tissue welding is also known to prevent platelet-platelet interaction and adhesion [3,4]. We show that human serum albumin in vitro significantly decreases the percentage of P-selectin–expressing platelets.

P-Selectin expression reflects not only platelet activation and participation in thrombogenesis but also their interaction with leukocytes [21]. Furthermore, P-selectin–expressing platelets are able to prolong survival of these leukocytes [22]. Thus, activated platelets initiate and propagate not only thrombus formation but also inflammatory reactions at the thrombus formation site. Albumin has the potential of preventing these reactions, because it reduces the proportion of P-selectin–expressing platelets.

To study P-selectin expression, we first compared methods of blood specimen collection. A small percentage of P-selectin–positive platelets was found in blood samples drawn by needle puncture. This number was increased in syringe-

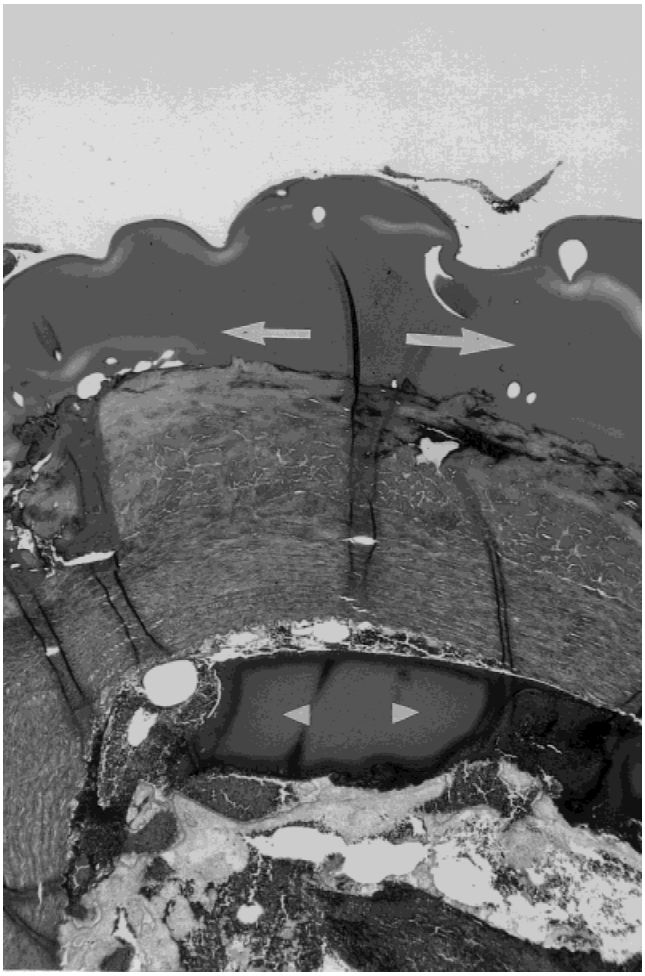

Fig. 6. Albumin is found at both the luminal and adventitial surfaces of the carotid artery. At 2 hours after repair, arteries were fixed in formalin, embedded in paraffin, and sectioned. Trichrome-stained sections reveal the presence of albumin (layers of albumin denoted by arrows) both at the adventitial surface (arrows) and at the luminal surface (arrowheads).

drawn aliquots. Therefore, all further samples were obtained by needle puncture.

We also examined what type of leukocytes form aggregates with platelets. According to the previously reported data, leukocytes that accumulate at platelet associated inflammation areas are predominantly polymorphonuclear cells and monocytes [23]. This is in agreement with our results showing that P-selectin–expressing platelets are found in complexes with large leukocytes, i.e., granulocytes and monocytes.

Finally, we tested the effect of albumin on platelet P-selectin expression in vivo, during laser repair of blood vessels in conjunction with albumin solder. We selected a time point of 2 hours, because we had previously compared wound strength of carotid arteries repaired with suture to those repaired with laser and albumin. In that study, there was no difference in the leak point pressure of the two types of repair [24]. The in vivo artery repair, both suture and laser energy assisted, was accompanied by a significant increase in platelet P-selectin expression. In suture repaired arteries, the percentage of P-selectin–positive platelets was significantly higher at the distal than at the proximal end. This finding suggests the ongoing platelet activation at the site of suture repair. In laser repaired arteries, the percentage of activated platelets was comparable at the proximal and distal ends. Moreover, the percentage of P-selectin–expressing platelets at distal ends of laser repaired arteries tended to be lower than at distal ends of suture repaired arteries. In these acute experiments, there was no platelet accumulation at the site of the anastomosis. However, the platelet activation that we have demonstrated could lead to future accumulation in chronic wound repairs. However, it is clear that not only suture, but also albumin-laser repair leads to a significant increase in P-selectin expression on platelets. It is possible that access of platelets to albumin in vivo differs from that in vitro. Furthermore, the concentration of albumin used in vitro may be higher than the local concentration of albumin used in laser welding. Newer methods of solder delivery with a higher concentration of albumin [25] might provide a higher amount of albumin at the site of repair, which would lead to a blockage of P-selectin expression. As shown in Figure 6, albumin can be found at both the luminal and adventitial surfaces of the blood vessel, suggesting that at least some portion of the albumin is in direct contact with the blood. This study also demonstrates that the use of laser energy in conjunction with albumin does not result in higher platelet activation than suture repair.

The mechanism of the albumin induced decrease in surface P-selectin expression is not clear. It might block this surface molecule by binding it, as in the case of previously reported CD43 [25]. Our results from the study with labeled albumin show that this protein binds the entire population of platelets. Thus, application of human fatty acid free albumin might prevent complications associated with over-expression of P-selectin on platelets. We conclude that albumin is a useful platelet activation inhibiting biomaterial and future research is needed to optimize its

platelet P-selectin blocking abilities during laser repair of blood vessels.

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